Molecular events accompanying Rous sarcoma virus rescue from rodent cells and the role of viral gene complementation

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Running title: Characterization of RSV rescue from rodent cells

Word count for the abstract: 217

Word count for the main text: 5843
Rodent cells transformed with avian Rous sarcoma virus (RSV) opened new ways to studying virus integration and expression in non-permissive cells. We were interested in the molecular changes accompanying fusion of RSV-transformed mammalian cells with avian cells leading to virus rescue and in enhancement of this process by retroviral gene products. Hamster RSV-transformed RSCh cell line was characterized as producing only a marginal amount of \textit{env} mRNA, no envelope glycoprotein and a small amount of unprocessed Gag protein. Viral unspliced genomic RNA egress from the nucleus was hampered and its stability decreased. Cell fusion of DF-1 chicken cell line with RSCh cells led to production of \textit{env} mRNA, envelope glycoprotein, and to processed Gag and virus-like particle formation. Proteosynthesis inhibition in DF-1 cells suppressed steps leading to virus rescue. Furthermore, new aberrantly spliced \textit{env} mRNA species were found in the RSCh cells. Finally, we demonstrated that virus rescue efficiency can be significantly increased by complementation with \textit{env} gene, highly expressed \textit{gag} gene, and the most by a helper virus infection. In summary, Env and Gag synthesis is increased after RSV-transformed hamster cell fusion with chicken fibroblasts and both proteins provided in \textit{trans} enhance RSV rescue. We conclude that the chicken fibroblast yields some factor(s) needed for RSV replication, particularly Env and Gag synthesis, in non-permissive rodent cells.
One of important issues in retrovirus heterotransmission is related to cellular factors that prevent virus replication. Rous sarcoma virus (RSV), a member of the avian sarcoma and leukemia family of retroviruses, is able to infect and transform mammalian cells; however, such transformed cells do not produce infectious virus particles. Using the well-defined model of RSV-transformed rodent cells we established that the lack of virus replication is due to the absence of chicken factor(s), which can be supplemented by cell fusion. Cell fusion with permissive chicken cells led to an increase in RNA splicing and nuclear export of specific viral mRNAs, as well as synthesis of respective viral proteins and production of virus-like particles. RSV rescue by cell fusion can be potentiated by in trans expression of viral genes in chicken cells. We conclude that rodent cells lack some chicken factor(s) required for proper viral RNA processing and viral protein synthesis.
Retrovirus functions have been systematically studied by delineation of the retroviral genome structure and its individual genes and functional domains. However, it turned out that the host cell can alter expression of such genes and domains. Cellular factors may act in a dominant negative way, efficiently suppressing viral functions in different steps of the virus replication cycle. Such factors have been isolated and characterized (1-3). The cell can also keep virus infection in check by the lack of cell functions required for virus replication. In such case, it is more demanding to characterize the set of functions involved. One of the first models for the latter situation was provided by some mammalian cell lines transformed with avian Rous sarcoma virus (RSV) strains. These cell lines (designated originally as virogenic) harbor the integrated retrovirus genome indefinitely in every tested clonal cell population as integrated provirus (4). However, the viral genome is not fully expressed and infectious virus production is not detectable. Such RSV-transformed cells can be forced to produce virus by cell fusion with permissive chicken fibroblasts (5), which was confirmed and extended (6-9). The RSV rescue studies also promoted HIV rescue experiments, which showed that despite adjusting rodent cells to early steps of HIV infection, these cells remained largely non-permissive with regard to infectious virus production. However, infectious HIV synthesis was triggered when such cells were fused with permissive human cells (10-12). This indicated that permissive cells provided some function, missing in non-permissive cells, that needs to be present in order to ensure full virus genome expression.

Despite that the cytological parameters of virus rescue have been clearly established and confirmed (5, 7, 13), we still lack the molecular insight into this process. For our study we employed the RSCh line of Chinese hamster fibroblasts transformed in vitro with the Schmidt-
Ruppin (SR) RSV strain, whose cytogenetic profile has been studied at regular intervals before, during and after transformation (13). This cell line has also been thoroughly tested for the absence of any infectious RSV production and employed in quantitative virus cell fusion experiments (5).

We show here that envelope messenger RNA (env mRNA) splicing and unspliced genomic RNA (gRNA) nuclear export as well as Env and Gag protein formation occur after fusion of non-permissive RSCh cells with chicken fibroblasts. We have demonstrated that cooperation of these molecular events is required for RSV virus rescue from non-permissive cells. We were also able to compare the SR-RSV splicing pattern with the previously tested Prague (PR) RSV strain in mammalian cells (14) and we found additional aberrant env mRNA splice variants. Furthermore, we have documented that virus rescue efficiency can be increased by complementation via cell fusion with Env- or Gag-producing cells. However, best results were achieved with chicken cells pre-infected with ALV helper virus. These results are discussed in relation to the general problem of cell factor involvement in infectious retrovirus formation.

MATERIALS AND METHODS

Cell cultures
RSCh is a Chinese hamster tumor cell line transformed with the Schmidt-Ruppin strain of RSV (SR-RSV-D). H-20 is a Syrian hamster cell line derived from a tumor induced by the Prague strain of RSV carrying only one provirus copy per genome (15). The DF-1 chicken cell line free of alpha endogenous retroviral (ev) loci was obtained from S. Hughes. Brown Leghorn (BL) chickens were selected for their sensitivity to RSV by Dr. Carr, who also kindly provided them to
our laboratory. Avian leukosis virus (ALV) infection in this close breed was eliminated. Chicken embryo fibroblasts (CEF) from these chickens were prepared by standard procedures from 10-day-old embryos and are denoted as CEF-BL. Japanese quail fibroblasts (QEF) were prepared from 8-day-old embryos. 16Q is the QEF cell line transformed by Bryan RSV strain lacking the envelope gene (BH-RSV) and was developed by H. Murphy (16). It represents a versatile tool for ALV env detection.

**Cell treatment and transfection**

Cells were grown in 1:1 DMEM and F12 medium (Life Technologies) supplemented with L-glutamine, 5% calf serum, 1 to 5% fetal calf serum, 1% chicken serum and 10% tryptose phosphate broth (Life Technologies).

Cell suspension was X-irradiated with 100 Gy using Wolf-Medizin Technik RTG T-200. No replicated cells survived as measured by control seeding of X-irradiated cells, which were followed for four weeks. Mitomycin C (Sigma) was applied for 2 hours in the amount of 10 μg per 1 ml medium. Cell cultures were then washed and left for 2 hours in mitomycin-free medium.

Two antibiotics, cycloheximide (Calbiochem) and puromycin (Sigma), were used for proteosynthesis inhibition. Cycloheximide was applied for 24 hours and puromycin for 15 hours, both in concentration 10 μg per ml.

Actinomycin D (Sigma) was employed for transcription inhibition in a concentration of 1 μg per ml.

Transfection was performed using the Polyplus protocol (Polyplus transfection). Cells were exposed to Optimem (Gibco) 1 hour before and during transfection (2 ml per 60 mm dish) and then 2 to 4 μg DNA per 60 mm dish was added. Plasmid containing the vesicular stomatitis virus
G glycoprotein region (pVSV-G) was obtained from Clontech. The pcGagPol construct was described previously (17).

**Cell fusion and infectious center assay**

For cell fusion we followed the standard polyethylene glycol (PEG) procedure using BioUltra PEG 6000 (Fluka). Briefly, irradiated or mitomycin-treated cells were mixed, and 12 hours after plating the cells were treated with 3.5 ml of 50% PEG for 45 s and rinsed three times with DMEM without serum. Under our experimental conditions, polynuclear cell formation varied between 27% and 30% of total cell counts after fusion as revealed by Hoechst 33342 (5 μM for 20 min) nuclear staining and phase contrast microscopy.

Infectious center assay was performed as described (5). Proliferating foci were counted within three weeks and virus titers were expressed as focus-forming units (FFU) per 1 ml of medium.

**Cell fractionation and RNA extraction**

Cell fractionation was performed according to (18). Cells were detached and washed in phosphate-buffered saline (PBS). The pellet was resuspended in 450 μl buffer RLN (50 mM Tris-HCl, pH 8.0; 140 mM NaCl; 1.5 mM MgCl2; 0.5% Nonidet P-40 substitute; 1,000 U/ml RNase inhibitor [Promega]; 1 mM dithiothreitol) and incubated for 5 min on ice. Debris and nuclei were pelleted (300 × g, 2 min, 4°C), and the cytoplasmic fraction was transferred to 1 ml of RNAzol (MRC). For RNA isolation from cell nuclei, the nuclear pellet was washed in 500 μl PBS, again pelleted for 3 min at 300 × g, and then resuspended in 1 ml of RNAzol. RNA was extracted from cultured cells and cell fractions with RNAzol according to the manufacturer's protocol.

**Quantitative and semi-quantitative RT-PCR**

RNA samples were treated with DNaseI (Roche) before RT-PCR to remove any contaminant DNA for 15 min in the M-MLV reverse transcriptase buffer (Promega). One microgram of RNA
was reversely transcribed into cDNA using random hexanucleotides and M-MLV reverse transcriptase (Promega) according to the manufacturer's protocol.

One μl of cDNA was used for the quantitative PCR (qPCR) based on the MESA GREEN qPCR MasterMix Plus for SYBR Assay Kit (Eurogentec) and a CFX96 system for qPCR detection (Bio-Rad). Quantifications of viral transcripts were performed with primers RSV-fw (CTTAGGAGGGCAGAAGCTGA) and unspliced_rv (GTTTTACACGCGGACGAAAT) for gRNA, RSV-fw and src_rv (GAGGCCACCAGCAGAGTC) for src mRNA, RSV-fw and envD_rv (TCGGAAATAGGAGACGGGATA) for env mRNA, dbl2-fw (GCCAGGGAACCTTTGGATTAGA) and src_rv for double spliced env_2 + cryptic spliced 2 (dbl2+cryptic2) mRNA, polend-fw (CAGCTGTGAAAAACAGGGACA) and src2_rv (GGGGTCCCTTAGGCTTGCTC) for cryptic spliced 1 + 2 mRNA, dbl3-fw (TGCTTCTAACTCCACGGAAACC) + src_rv for double spliced env_3 + cryptic spliced 3 (dbl3+cryptic3) mRNA. Transcripts were either normalized to total viral RNA with primers srcdel_fw (GACTGAGCTGACCACCAAGG) and srcdel_rv (GCACTACTCAGCGACCTCCA) (results are presented as the percentage of total viral RNA) or to the mammalian glyceraldehyde-3-phosphate dehydrogenase (mGAPDH) housekeeping gene transcripts with primers mGAPDH-fw (AACTTGGCATTGTGGAAGG) and mGAPDH_rv (ATCCACAGTCTTCTGGTGTTG), or to chicken glyceraldehyde-3-phosphate dehydrogenase (chGAPDH) with primers chGAPDH-fw (CATCGTGACCACCAACTG) and chGAPDH_rv (CGCTGGGATGATGTCTTG). The volume of the reaction mixture was 20 μl with 400 nM final concentration of each primer. The external standards for gRNA, env, src, dbl2+cryptic2, cryptic2, dbl3+cryptic3, total viral RNA and mGAPDH were constructed by PCR using RSCh cDNA and transcript-specific primer sets. Resulting PCR fragments were cloned into pGEM-T
Easy (Promega) and verified by sequencing. Calibration curves were prepared by PCR of diluted plasmid samples ranging from $10^5$ to $10^7$ copies per reaction. Subcellular fractionation was also controlled with SYBR green-based RT-PCR, using primers preGAPDH_fw (TCATCCTGCCAGCAGTGG) and preGAPDH_rv (CAGAGGCCAGGGAGTGAGGTC), homologous to intron 5 of the unprocessed pre-mRNA of the gene (pre-GAPDH RNA). One μg of extracted nuclear and cytoplasmic RNAs was subjected to RT-qPCR. This allowed calculation of the percentage of pre-GAPDH RNA in the cytoplasm relative to the nuclear pre-GAPDH RNA level. Cycling conditions for gRNA, src, dbl2, dbl3, total viral RNA and GAPDH were 5 min at 95°C, 40 cycles of 15 s at 95°C, 20 s at 61°C, 15 s at 72°C, for env 5 min at 95°C, 40 cycles of 15 s at 95°C, 20 s at 59°C, 30 s at 72°C, for cryptic1+2 5 min at 95°C, 40 cycles of 15 s at 95°C, 20 s at 62°C, 40 s at 72°C, and for preGAPDH 5 min at 95°C, 40 cycles of 15 s at 95°C, 20 s at 63°C, 15 s at 72°C. We used water and samples without reverse transcriptase as negative controls.

For semi-quantitative analysis of endogenous viral mRNAs, cDNA was used as a template, and primers RSV_fw and unspl_rv for the gag region and chENV233fwd (ACGGATTCTGCCTCTCTACACA) and chENV1046rev (TTCCCTGCATGCAGGCATCCC) for the env region were employed. For identification of aberrant splicing of SR-RSV we used two pair of primers: RSV_fw and src_rv, polend_fw and src2_rv.

**Immunofluorescence**

For Env product detection we employed immunoadhesin Tvb-mIgG. The tvbSI gene was equipped with the mouse IgG gene (gift from M.J. Federspiel), which was incorporated into DF-1 cells, where it produced the respective soluble immunoadhesin (19). The Gag product was detected by mouse anti-p27 monoclonal antibody (gift from E. Humphries, West Virginia...
Immunofluorescence microscopy was performed as described previously (20). Briefly, cells grown on coverslips were fixed for 20 min in 3% formaldehyde. Fixed cells were incubated for 60 min with Tvb-mIgG supernatant diluted 1:5 or anti-p27 antibody diluted 1:500. Binding of immunoadhesin Tvb-mIgG or anti-p27 antibody with their respective \textit{env} or \textit{gag} gene product was revealed by staining with Cy3-conjugated anti-mouse antibody (Jackson Immunoresearch Laboratories, West Grove, PA; dilution 1:1000). The preparations were mounted in MOWIOL 4-88 (Calbiochem, San Diego, CA) supplemented with 4,6-diamidino-2-phenylindole (DAPI, Sigma) to label cell nuclei and examined with an Olympus AX-70 Provis microscope.

**Western blot**

Cells and cell-free supernatants were harvested four days after fusion. The cells were lysed in SB buffer (2% sodium dodecyl sulfate [SDS], 10% glycerol, 0.625M Tris-Cl, pH 6.8, 0.1% bromophenol blue, 5% β-mercaptoethanol). The viral particles were pelleted from the cell-free supernatant by ultracentrifugation through 25% sucrose cushion at 32000 rpm for 1.5 hour in a Beckman SW41Ti rotor. The viral particles were lysed in SB buffer. The lysate of cells and viral particles was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and separated protein bands were transferred to PVDF membrane (Amersham). Rabbit anti-p27 IgG fraction conjugated to horseradish peroxidase (Charles River) was used at a 1:750 dilution to examine the Gag polyprotein in Western blot assay. LumiGLO chemiluminescent substrate (Cell Signaling) was used to reveal the protein bands of unprocessed and processed Gag polyprotein. Equal protein loading and transfer was verified by performing immunodetection of GAPDH (dilution 1:4000, Invitrogen) on the same membrane.
RESULTS

Reevaluation of RSV-transformed rodent cells and selected chicken cells used for virus rescue

In order to verify our stock of RSCh cells we performed karyological analysis. The cells contained typical Chinese hamster chromosomes and counting their numbers revealed that the highest representation of karyotypes corresponded to pseudodiploid counts – 22 chromosomes (data not given), which agrees with our previous observation that repeatedly passaged RSCh cells show a clear tendency to acquire aneuploid karyotype (13). As has been documented (21), RSCh harbor fully functioning provirus that can be transmitted to CEF-BL by DNA transfection, which historically represents the second independent and successful transfection experiment. Using qPCR for comparison of RSCh with H-20 cells carrying only one provirus copy (15) we found that RSCh cells contain an almost equal amount of proviral DNA (Fig. 1A). By terminal dilution, several monocellular clones were established, and one of them was employed in further experiments.

For virus rescue experiments we employed stable line of chicken fibroblasts DF-1 to eliminate any effect of endogenous genes. Previously used primary chicken fibroblasts CEF-BL contain four endogenous retroviral loci (ev) (22), while DF-1 are regarded as free of ev loci (23). Because we wanted to exclude the possibility that some viral protein synthesis activity remained undetected, we performed additional control using RT-PCR with primers covering the conservative env region (24) and gag leader. As documented in Fig. 1B, in contrast to CEF-BL, DF-1 cells produced no expected env fragment. Similarly, no gag gene transcript was detected in DF-1 cells (Fig. 1C).
We retested the virus rescue capacity of RSCh cells by fusion with chicken CEF-BL using PEG. Then we established that cell line DF-1 can substitute for primary chicken fibroblasts CEF-BL used so far for virus rescue. RSCh cells, either mitomycin-treated or X-irradiated, fused with DF-1 after four days produced 15 to 18 FFU/ml (focus-forming units) of virus in the culture medium (filtered or unfiltered) in contrast to untreated, treated or self-fused RSCh, which produced no infectious virus. In the second step, we successfully isolated three foci, sub-cultured them on CEF-BL, and supernatants were tested in the focus assay seven days later. All cultures produced $10^4$ to $10^5$ FFU/ml of virus.

**Viral RNA splicing and synthesis in non-permissive RSCh cells and fused cells**

As we learned from the past experience, cell fusion between non-permissive RSV-transformed rodent cells and permissive chicken fibroblasts provides an efficient way to rescue infectious virus. Therefore, we were interested in the course of the events accompanying this process of virus rescue. Because of our previous finding that in non-permissive RSV-transformed rodent cells *env* mRNA was undetectable by Northern blotting (15) and gRNA signals were far less intensive than *src* mRNA, we focused on RNA quantification using RT-qPCR. In order to measure individual viral RNA species we designed a set of primers schematically represented in Fig. 2A. They allowed us to quantify different viral mRNA species.

As shown in Fig. 2B, control chicken DF-1 cells infected with SR-RSV-D synthesized mainly gRNA (62 %), followed by *env* (23 %) and *src* mRNA (15 %). Contrary to that, the main RSCh viral RNA species was represented by *src* mRNA (66 %), followed by a smaller gRNA amount (20 %), whereas *env* mRNA fell almost beyond detection. The situation changed after RSCh fusion with DF-1, when the *env* transcript was clearly detected, representing about 7.5 % of total viral RNA. The *src* mRNA level was significantly decreased and the gRNA amount did
not change after fusion. Mitomycin treatment or X-radiation used to stop replication after fusion did not change the splicing pattern of regularly spliced viral mRNAs.

**New aberrantly spliced viral mRNA species in RSCh cells**

When we quantified *env* mRNA in RSCh cells, we encountered different expression levels depending on the primer position. When the reverse primer was positioned close to the *env* splice acceptor, the amount of *env* mRNA was higher. This indicated that aberrant splicing using cryptic splicing sites might have been involved. Because aberrant splicing of the PR-RSV strain has been described (25), we decided to find out whether SR-RSV is processed in the same way. Using two pairs of primers we identified five aberrantly spliced viral mRNAs species — cryptic double-spliced *env* mRNA 1 and 2 (Dbl1, Dbl2) and cryptic single-spliced mRNA 1, 2 and 3 (Fig. 3A, 3B). Aberrantly spliced RNAs were separated, extracted from the gel and sequenced. Three different cryptic SD sites were identified in the 5' half of the *env* gene (Fig. 3A), thus splicing products coding for truncated proteins. Dbl1 and Dbl2 arose by normal splicing from the splicing donor (SD) site in *gag* to the splicing acceptor (SA) site upstream of the *env* gene and by aberrant splicing from cryptic SD sites to SA between the *env* and *src* genes. Cryptic spliced 1, 2, and 3 RNAs differ from Dbl by the absence of *gag-env* splicing. Their primary nucleotide sequence fitted with their position given in Fig. 3A with the exception of Dbl3, which was not detected by PCR and therefore its structure is only putative. Furthermore, cryptic SD sites utilized by splice variants were evaluated by their capability to form H-bonds with U1 snRNA (26). H-bond scores of cryptic sites 1, 2, and 3 were found to be 15, 16.9, and 13.8, respectively (http://www.uni-duesseldorf.de/rna/html/h-bond-score.php), which corresponds to 14 hydrogen bonds found as a proper consensus. In addition, aberrantly spliced RNA representation is in accordance with their H-bond scores (Fig. 3B, 3C). The amount of aberrantly spliced viral RNAs measured by RT-
qPCR in RSCh cells exceeded that in chicken cells, reaching 5.6 % of total viral RNA in the former and 1.4 % in the latter cells (Fig. 3C). We point out that we used RSCh cell line in our study, which was shown to contain only one proviral copy, and therefore cryptic splicing cannot result from additional aberrant provirus transcription.

For the cell fusion experiment we employed cells exposed to genotoxic treatments such as mitomycin C and X-irradiation in order to prevent cell replication after fusion. This was not without consequences for splicing. Aberrant splicing, especially Dbl2+Cryptic2, was highly increased (Fig. 3C). This is not surprising in the light of other studies performed on other models (27, 28).

In summary, we identified new cryptic SD sites in the SR-RSV-D env and observed the respective aberrantly spliced mRNA species in RSCh cells. The level of aberrant splicing significantly increased after the genotoxic treatment, but changed only moderately after fusion with chicken cells.

**Rescue of env mRNA and envelope glycoprotein after fusion with permissive cells**

Because comparison of viral mRNA species in RSV-transformed hamster cells and in RSV-transformed hamster cells fused with chicken cells revealed a striking difference in the env mRNA level, which increased 7-fold on the fourth day after fusion (Fig. 2B), we decided to study env mRNA and respective protein synthesis in more detail. First, we analyzed the time course of viral envelope glycoprotein synthesis using the immunoadhesin, Tvb/d receptor equipped with mIgG domain, detecting the respective glycoprotein. Fig. 4 demonstrates multinuclear cells exhibiting fluorescence on the fourth day after fusion. The positive signal was achieved in 30 % of polykaryons, whereas cells containing single nuclei were negative. The kinetics of envelope
glycoprotein formation after fusion is given in Fig. 5A. There is a good correlation between the
increase in proportion of positively stained multinuclear cells (bar graph) and env mRNA
synthesis (line graph) as assayed by RT-qPCR.

Furthermore, we pre-treated partner cells separately with protein synthesis inhibitors
(puromycin or cycloheximide) before fusion to see whether the decreased amount of proteins
with shorter half-life influences the effect of fusion on splicing. We observed that the increase in
env mRNA synthesis after fusion was significantly lower when the permissive chicken partner
cells were exposed to puromycin or cycloheximide (Fig. 5B). We assume that the level of the
factor required for env mRNA synthesis in heterokaryons decreased due to proteosynthesis
inhibition in chicken fibroblasts, and thus the fusion was not so effective in triggering env mRNA
splicing. Treatment of RSCh cells with proteosynthesis inhibitors did not produce any significant
effect on the env mRNA level after fusion. This indicates that at least for env mRNA synthesis,
some chicken protein factors are required.

Nuclear export and stability of gRNA

Although we did not detect any considerable change in the total amount of gRNA after
fusion with chicken cells, cell fusion might have exerted its influence even at a more subtle level
related to gRNA egress from the nucleus. We therefore compared gRNA export in RSCh versus
the positive control 16Q (quail cells harboring defective RSV genome producing gRNA and src
mRNA). In the latter case, cytoplasmic gRNA reached 20 % of nuclear gRNA in contrast to
RSCh, where gRNA representation in the same fraction dropped 10-fold (Fig. 6A). Based on a
repeated experiment, in which we measured the gRNA quantity present in the cytoplasmic
fraction, we obtained evidence that the RNA egress from RSCh nuclei increased three times after
fusion with DF-1 cells (Fig. 6B). The cytoplasmic fraction purity was also tested and it was
established that contamination with nuclear RNA did not exceed 4% (Fig. 6C) and did not change after the fusion (Fig. 6D).

Finally, we checked gRNA stability after RNA synthesis inhibition using exposure to actinomycin D. In these experiments we compared RSCh cells with SR-RSV-infected DF-1 cells and found that in hamster cells the gRNA stability dropped down during the first hours after actinomycin addition, while in chicken cells gRNA remained stable (Fig. 6E).

These results show that gRNA in RSCh cells is unstable, its level is low, and although its total amount is not altered by fusion with chicken cells, gRNA nuclear export significantly increases.

**Gag expression after cell fusion**

The viral gRNA also serves as the mRNA encoding the Gag polyprotein. Thus, we decided to confirm the above-mentioned data concerning gRNA with direct Gag protein measurement using immunology approaches. Using antisera against Gag for RSCh cell staining, weak fluorescence diffused in the cytoplasm was found. The fluorescence intensity was clearly lower when compared with that obtained in heterokaryons (Fig. 7A).

By Western blotting procedure we again observed that RSCh cell lysates produce almost undetectable amounts of the Gag precursor protein (Fig. 7B). Furthermore, when RSCh cells were fused with DF-1, expression of Gag protein increased and the regularly processed Gag p27 product appeared. Employing Western blotting we also verified the presence of processed Gag synthesis products in DF-1 cells transfected with pcGagPol, DF-1 cells infected with the ALV helper viruses and 16Q cell line (Fig. 7C).

Finally, in order to check the release of virus like particles (VLP), the supernatant from RSCh cell culture was spun through sucrose cushion and the sediment was also subjected to Gag
analysis. As documented in Fig. 7B, the sediment obtained from RSCh was devoid of the Gag presence. Not surprisingly, the sediment from RSCh cells fused with DF-1 gave rise to the clear p27 band, thus reinforcing the importance of permissive cells for virus particle formation.

We have shown that fusion of mammalian RSCh cells with chicken cells induces not only RSV env mRNA splicing, Env glycoprotein synthesis and gRNA nuclear export, but also Gag protein synthesis and processing as well as VLP formation and release from the fused cells.

**Complementation with env and gag genes**

Our results pointed to the envelope glycoprotein and Gag protein importance in the facilitation of virus rescue. We therefore decided to test env and gag genes complementation in RSV rescue experiments. In order to test virus rescue efficiency, RSCh cells were fused with DF-1 cells producing Env or Gag, fused cells were plated on indicator cells and the rescued transforming virus formation was measured as foci by infectious center assay. We employed CEF-BL cells as indicator, because DF-1 as a modified and stabilized cell line cannot be used for focus screening (our and others repeated observations).

As Env we employed vesicular stomatitis virus G glycoprotein (VSV-G) known to efficiently provide a heterologous envelope to retroviruses (29). As given in Fig. 8A, RSCh fused with VSV-G-transfected DF-1 produced 5- times more foci than RSCh fused with untransfected DF-1 cells. In order to examine the influence of processed Gag protein on virus rescue we transfected DF-1 cells with the plasmid pcGagPol containing a selectable marker and the gag gene. After transfection and selection, DF-1 cells expressed low amounts of Gag (Fig. 7C). After fusion with RSCh cells we observed no increase in RSV rescue in comparison with untransfected
DF-1 cells. These results indicate that a low level of Gag cannot complement RSV replication in non-permissive mammalian cells.

In order to check whether higher amounts of Gag can influence virus rescue efficiency we used the 16Q cell line, which is derived from QEF cells transformed by BH-RSV (RSV strain lacking the env gene) and therefore rich in Gag protein (Fig. 7C). RSCh fusion with quail QEF cells was slightly less effective than fusion with DF-1 cells (Fig 8A), but when we employed 16Q as partner cells, the virus rescue efficiency markedly increased. In order to confirm our results with VSV-G complementation, RSCh cells were fused with VSV-G-transfected QEF cells. Virus rescue efficiency was 13-times enhanced (Fig 8A).

A direct way to equip mammalian cells with both env and gag gene products is provided by their fusion with ALV-infected partner cells. In such a way DF-1 rescue activity in fusion experiments with RSCh was increased by two orders of magnitude when DF-1 cells were exposed to RAV-1, a helper virus replicating in such cells (Fig. 8A).

Mixture of RSCh and DF-1 cells without PEG fusion was tested to determine control background of virus rescue caused likely by spontaneous cell fusions. Mixture of 10^5 cells resulted in none or one focus, which correspond with previously published data (5). Furthermore, we tested infectious virus formation in culture media from the tested cells (Fig. 8B). No virus activity was recorded with the exception of positive control represented by 16Q cells transfected with VSV-G, which as expected produced 6×10^3 FFU/ml.

In summary, these findings show that env gene complementation can significantly increase virus rescue efficiency. The increase after gag gene complementation was evident only
in the case of high expression of this gene. The best results were achieved when RSCh cells were fused with chicken cells infected with helper virus providing both env and gag gene products.

DISCUSSION

For decades RSV and avian leukemia viruses have been a cornerstone of retrovirus research (30). Furthermore, RSV was shown to transgress class barriers under experimental conditions, which produced new challenges related to provirus integration, its expression and infectious virus rescue (31-33). In this report we concentrated on the molecular characterization of RSV-transformed rodent cell line RSCh and the consequences of RSCh cell fusion with avian cells leading to infectious RSV rescue. We are aware of the fact that virus rescue is only one side of a more general problem involving provirus expression in heterologous cells, including involvement of known cellular factors. This issue was thoroughly upgraded (34) and we shall return to it in our next study.

In order to extend and update the model of SR-RSV-transformed rodent cells we selected the original well-characterized RSCh cell line, which had been studied systematically and quantitatively in a series of previous communications (5, 13, 33). Our present data document that this tumor cell line produces very low amounts of env mRNA, confirming our previous Northern blot analysis of three rodent tumor cell lines (15). However, when fused with DF-1 chicken cells lacking endogenous alpharetroviral loci, both env mRNA and glycoprotein synthesis is initiated soon. Similarly, Gag protein synthesis is increased, its regular cleavage takes place, and in the tissue culture supernatant fraction corresponding to VLP particles the processed Gag is present. In such a way, the main molecular steps required for virus rescue are accomplished thanks to
rodent cell complementation with chicken cell factor(s). The complementation hypothesis is supported by the finding that protein synthesis inhibitors block increase of the env mRNA level when applied to DF-1 cells and not to RSCh cells before the fusion.

One of the factors responsible for the env mRNA threshold synthesis might be related to aberrant splicing. We found that quantified aberrant splicing of the env gene represents only 5.6% of total viral RNAs in non-permissive cells, but is also detectable in permissive cells, where it reaches 1.3%. Aberrantly spliced RNAs utilized different cryptic splice donor (SD) sites and the same regular splice acceptor (SA) site. The splice variants designated as Dbl1 and Cryptic1 have already been described in PR-RSV (25), but the new ones – Dbl2, Cryptic2 and Cryptic3 – are characteristic for SR-RSV, where a single nucleotide substitution created a new cryptic SD site. From both our data and (25), it seems highly unlikely that aberrant splicing alone might play any decisive role in diminishing the pool of full-length env transcripts. Rather, the env gene represents most recent innovation in the retrovirus evolution, and therefore we can expect its variability.

Quite a lot of attention has been paid to the molecular mechanisms playing a role in RSV gRNA export from the cell nucleus to the cytoplasm. Unique elements called direct repeats (DRs) constituting the 3’ end of the viral genome were shown to represent a genomic “mark” licensing gRNA export and stability. RSV contains two DRs, which are structurally highly similar. A series of following papers using various upstream or downstream DR modifications provided data indicating their role in the virus life cycle (35-41). The most convincing are observations obtained in viruses lacking both DRs that provided evidence for the drop in gRNA stability, egress, and possibly also in the Gag product synthesis (35). Our study of RSCh cells revealed some similarity to viruses lacking DR. This concerns gRNA instability, decreased export from
the nucleus and low efficiency of Gag product synthesis. Furthermore, as reported (35) and
confirmed in our experiments (unpublished), DR deletion does not impart any effect on the RSV
replication in rodent cells. On the other hand, it was demonstrated that DR facilitates gRNA
egress from the nucleus in mammalian cells (42). Still, there remains the possibility that rodent
cells are devoid of some factor(s) needed for interaction with DRs or produce an inhibitor
paralyzing the DR function, especially at later stages of gRNA processing that follow its export.
Other factors such as Gag import in the nucleus (43) and non-structural Gag protein cleavage
product p10 equipped with the nucleus localizing signal (44) should also be taken into account.

Interesting results were obtained by studying consequences of partner cell
complementation with gag and env gene products or helper virus infection. So far it is known that
RSV-transformed rodent cells are not complemented when different independently obtained cell
lines are fused one with the other (45). Similarly it was revealed that infection of transformed
cells with rodent retroviruses does not lead to RSV rescue in spite of the fact that rodent
retroviruses complete their replication cycle (46). Here we utilized a different strategy in which
we did not complement non-permissive rodent, but permissive avian partner cells with gag, env,
or both gene products. Under such conditions a clear increase in RSV yield after cell fusion
occurred; however, it was dependent on the degree of complementing gene expression. This
supported our conclusion that the deficiency of rodent cells lies in part in their inability to
synthesize or process gag or env gene products. This deficiency can be diminished by supplying
these products in trans. However this trans-complementation needs to be supported by fusion
with permissive cells. Gag or env gene products are not sufficient to ensure virus rescue in non-
permissive rodent cells by themselves, but they can increase RSV rescue efficiency after fusion
with chicken cells. This indicates that not only complementation with gag and env gene products,
but also other factors from permissive cells are required.

We are finishing with the quotation from a recent HIV review stating that the long-
standing puzzle of cell type-dependent requirement for viral replication remains an exciting area
of research (47). In the case of RSV strains, this area has been with us for long years but acquired
only humble attention.

ACKNOWLEDGMENTS

We thank J. Konvalinka for his sincere interest in our work and material help. We are
thankful to K. Michalová and J. Březinová for chromosomal preparations, D. Staněk and D.
Elleder for interesting discussions. We are also obliged to K. Beemon for providing us with DR
mutants, as well as to H.G. Krauslich for his 4xCTE construct and B.R. Cullen for his Tap clones.
The technical assistance of L. Mikušová and D. Kučerová is also acknowledged.

Anna Lounková is a PhD student registered at Faculty of Science, Charles University and
her work was partly funded by the Charles University Grant Agency (Project no. 43-251309).
This project was supported by the Czech Science Foundation (Projects nos. P502-11-2207, P302-
12-1673). The work was institutionally supported by RVO: 68378050.

REFERENCES


FIGURE LEGENDS
FIG. 1. Characterization of hamster and avian cells used for virus rescue. (A) The number of RSV proviral copies in hamster RSCh cells was compared with that in H-20 cells. Chromosomal DNA was isolated from RSCh and H-20 cells. Twenty-five ng of DNA was subjected to qPCR and using virus-specific primers (SrcDel_fw and SrcDel_rv) $c_q$ values were measured and compared. Results represent the mean from triplicate samples ± SD. (B, C) Expression of retroviral mRNAs in DF-1 and CEF-BL cells was analyzed by RT-PCR. For $env$ mRNA detection (B) we employed primers designed to detect the conservative $env$ region (chENV233fwd and chENV1046rev) and for gRNA (C), we used primers covering the leader and left part of the $gag$ gene (RSV-fw and unspl_rv).

FIG. 2. Comparison of different viral RNAs synthesis in RSV-transformed hamster RSCh cells, chicken DF-1 cells infected SR-RSV and RSCh cells fused with DF-1 cells. (A) Schematic summary of SR-RSV-D genome and its transcripts is depicted. Different viral RNA species are shown together with the positions of primers for splicing-specific qPCR. (B) The amounts of viral RNAs were quantified. DF-1 cells were infected by SR-RSV-D. RSCh were exposed before fusion to genotoxic agent mitomycin or X-radiation. The fourth day after fusion total RNA was isolated. cDNAs synthesized by RT from the RNA samples were measured by qPCR. The amounts of viral mRNAs are given as percentages of total viral RNA. Respective primers that were employed are indicated above. Error bars represent standard deviations. Results are from three independent experiments, each in two parallels. The significance of differences between RSCh, RSCh cells exposed to mitomycin/X-ray and RSCh mit/X ray fused with chicken DF-1 cells are marked by asterisks (* $p = 0.01$-$0.001$, ** $p < 0.001$).
FIG. 3. Identification of cryptic spliced viral mRNA species in RSV-transformed hamster RSCh cells, chicken DF-1 cells infected with SR-RSV and RSCh cells fused with DF-1 cells. (A) Schematic representation of RSV proviral DNA with depicted splicing sites (splicing donor site – SD, splicing acceptor site – SA) and the structure of newly defined aberrantly spliced viral mRNAs is shown. Asterisk indicates cryptic splicing donor sites 1, 2, 3 at nt 5222, 5348, 5805 according to [GenBank: D10652.1], respectively. Position of PCR primers and product sizes are shown. Position of primers used for qPCR is depicted bellow. Dbl3 was not detected and its structure is only putative. (B) RSCh cells were exposed to mitomycin before fusion. Total RNA was isolated fourth day after fusion and then subjected to RT-PCR. Amplified PCR products obtained using the described primer sets were visualized in ethidium bromide-stained agarose gels. Size markers in base pairs are indicated on the left. (C) The amounts of cryptic viral mRNAs were quantified. DF-1 cells were infected by SR-RSV-D. RSCh were exposed before fusion to genotoxic agent mitomycin or X-radiation. The fourth day after fusion total RNA was isolated. cDNAs synthesized by RT from the RNA samples were measured by qPCR. The amounts of viral mRNAs are given as percentages of total viral RNA. Respective primers that were employed are indicated above. Error bars represent standard deviations. Results are from four independent experiments, each in two parallels. Significant differences between RSCh, RSCh cells exposed to mitomycin/X-ray and RSCh mit/X ray fused with chicken DF-1 cells are marked by asterisks (* p = 0.01-0.001, ** p < 0.001).
FIG. 4. RSCh x DF-1 polykaryons producing envelope glycoprotein. The 1:1 mixture of mitomycin-treated RSCh and DF-1 cells was fused. Cells were fixed on the fourth day after fusion and Env glycoprotein was detected by immunoadhesin Tvb-mIgG and visualized by goat anti-mouse antibody labeled with Cy3 (red). Nuclear DNA was stained with DAPI (blue). Polykaryons producing envelope glycoprotein are marked by arrows.

FIG. 5. Expression of env mRNA depending on the time after fusion and presence of proteosynthesis inhibitors in partner cells. (A) The expression level of envelope glycoprotein, env mRNA and three other viral mRNAs was determined during four days after the fusion. Line graph: the level of four different viral mRNAs (gRNA, src mRNA, env mRNA and dbl2+cryptic2 mRNA) was measured by RT-qPCR. Ratios of individual viral mRNAs to total viral RNA relative to RSCh mit were calculated. Error bars indicate standard deviations. Results are from two independent experiments. Bar graph: Determination of the relative amount of viral envelope glycoprotein-positive cells. At least 500 polykaryon cells were counted for each sample. (B) In two independent experiments, RSCh or DF-1 cells were treated before fusion, in addition to mitomycin, with inhibitors of proteosynthesis, puromycin (10 μg/ml, 15 hours) or cycloheximide (10 μg/ml, 24 hours). The second and fourth day after fusion the cells were lysed, total RNA was isolated and cDNA was prepared. The amount of env mRNA was measured by RT-qPCR.

FIG. 6. Nuclear export and stability of gRNA. (A) Viral gRNA was quantified in cytoplasmic and nuclear fractions. The amount of gRNA was determined in fractionated RSCh and 16Q cells by RT-qPCR. The cytoplasmic RNA levels are given as percentages of nuclear RNA levels. The
mean values ± SD for two to three independent experiments are shown. (B) The gRNA level in the cytoplasmic fraction of RSCh cells after mitomycin treatment and fusion with DF-1 cells was analyzed three or four days after the fusion. Asterisk indicates that the increase of gRNA after fusion was statistically significant (p<0.01). The data represents mean ± SD for three independent experiments. (C) The purity of isolated fraction is shown by preGAPDH levels. Cellular pre-mRNA of GAPDH in RSCh was detected via RT-qPCR. The cytoplasmic RNA levels are given as percentages of nuclear RNA levels. The results are means ± SD of three separated experiments. (D) The purity of RSCh cytoplasmic fractions after mitomycin treatment and fusion with DF-1 cells was measured by preGAPDH in three independent experiments. (E) The stability of viral RNAs in DF-1 cells infected with SR-RSV-D and RSCh cells was measured. Cells were incubated with actinomycin D (1 μg/ml) for each designed time period followed by RNA isolation and RT-qPCR. Graphs show levels of viral mRNAs at various time points after addition of actinomycin D, presented as the percentage of the RNA level at the time of addition of actinomycin D. Viral RNAs were normalized to GAPDH mRNA levels. The data represent the mean ± SD of three independent experiments.

FIG. 7. Gag expression. (A) Determination of protein Gag by immunofluorescence labeling in RSCh cells and RSCh cells fused with DF-1 cells. Cells were fixed on the fourth day after fusion and stained with anti-p27 antibody and Cy3-conjugated secondary antibody. (B) Expression of Gag precursor Pr76 and product p27 was determined by Western blot analysis with anti-p27 antibody of total cell lysates and VLPs. Comparison of Gag production in RSCh cells and RSCh cells mixed with DF-1 cells with or without fusion is shown. (C) Gag expression was analyzed by...
Western blot with anti-p27 antibody in chicken DF-1 cells with or without pre-infection with helper virus or transfection with pcGagPol, quail QEF and 16Q cells.

FIG. 8. Virus rescue efficiency after complementation with Gag, Env, or both. RSCh were complemented by fusion with avian cells synthesizing either VSV-G or Gag, or infected with helper virus. The amount of $10^4$ fused cells was plated on indicator cells and after 21 days, infection centers were counted. The data shows mean ± SD of two to four independent experiments and differences after complementation are depicted. (A) Virus rescued after RSCh cells fusion with chicken cells: DF-1, VSV-G-transfected DF-1, DF-1 transfected with pcGagPol, RAV-1-infected DF-1, and quail cells: QEF, VSV-G-transfected QEF and 16Q was tested on CEF-BL cells. DF-1 and QEF cells were transfected with VSV-G one day before fusion. RSCh mixed with DF-1 without fusion were used as a negative control. (B) Tissue culture fluid (sup) taken the second day after VSV-G transfection of RSCh and 16Q cells as well as untransfected RSCh, 16Q supernatants were centrifuged ($13000 \times g$, 20 min, 4°C) and tested in the focus assay on two parallel dishes on CEF-BL.